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Time-resolved fluoroimmunoassay for quantitative determination of tylosin and tilmicosin in edible animal tissues

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To quantitatively determine tylosin and tilmicosin in edible animal tissues, a time-resolved fluoroimmunoassay (TRFIA) has been developed and validated. For this purpose, desmycosin-*O*-carboxymethoxylamine-BSA was fixed onto microtiter plates, standards and samples were loaded and, finally, diluted europium-labeled anti-tylosin antibodies were added. Results show that the limit of detection for tylosin was 0.03 ng mL^{-1} and that for tilmicosin was 0.05 ng mL^{-1}. The recoveries were 73.6% to 120.5%, with coefficients of variation below 15.6% in various biological matrices spiked with tylosin and tilmicosin at concentrations of 50–200 ng g^{-1}. There was good correlation (R^2>0.99) between the TRFIA, an enzyme-linked immunosorbent assay and high performance liquid chromatography data. In conclusion, the new TRFIA is applicable to the detection of tylosin and tilmicosin and is an effective and economical method that will enable high-throughput sample screening. The method is expected to be widely applicable.

tylosin, tilmicosin, monoclonal antibody, time-resolved fluoroimmunoassay, edible tissues

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Tylosin, a macrolide antibiotic, is extensively applied in veterinary medicine because it is strongly resistant to most of Gram-positive and -negative bacteria, as well as *Mycoplasma* spp. and *Chlamydia* spp. [1]. Tilmicosin, (20-deoxo-20-(3,5-dimethyl-piperidin-1-yl)-desmycosin, is a semisynthetic macrolide antibiotic with a spectrum of microbiological activity against *Pasteurella* spp., *Mycoplasma* spp., and a variety of Gram-positive organisms [2]. For these reasons, tylosin and tilmicosin are widely employed to treat a broad range of infections in domestic animals, especially respiratory diseases. The residues of these antibiotics may stay in the edible tissue of animals when misused, which may trigger allergies and select for resistance. Therefore, the use of tylosin and tilmicosin in feed additives was banned in the European Union in 1999 [3]. Within China, maximum residue limits (MRLs) have also been established for tylosin and tilmicosin. Therefore, there is a need to develop a simple and efficient detection method to screen tylosin and tilmicosin antibiotics in food producing animals.

For animal product safety control, several detection methods for tylosin and tilmicosin residues have been developed in recent years, including high performance liquid chromatography (HPLC) [4–8], thin-layer chromatography [9], HPLC coupled with mass spectrometry [10–15], and microbiological assays [4,16]. However, expensive equipment, skilled technicians and complicated sample pretreatments are required for those methods, making them unsuitable for high throughput field detection or routine screening. The microbiological assay is lengthy and insufficiently specific for analytical purposes. Immunoassays are valuable as rapid, low cost and high throughput tests for rapid screening of a large number of samples. A number of researchers have established enzyme-linked immunosorbent assays (ELISAs) for tylosin and tilmicosin [1,17–20]. ELISA is used frequently because it is highly sensitive, portable and suitable for large-scale use but it is imprecise and positively biased

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because of matrix effects [21,22].

A highly sensitive, dual-label, time-resolved fluoroimmunoassay (TRFIA) that can simultaneously detect tylosin and tilmicosin is described in this paper, which offers advantages of cost and time efficiency, allowing simplified assay protocols and small sample volumes [22]. Because of the unique fluorescent properties of lanthanide chelates, which have the advantages of high quantum yields, narrowband emission peaks, long Stoke's shifts and fluorescence lifetimes, TRFIA is distinct from conventional fluorescent dyes. The half-life of background fluorescence is very short so the long duration of lanthanide chelate emission allows longer measurement, which distinguishes specific fluorescence from the background. Thus, the sensitivity of a TRFIA is higher than that of conventional methods, which lessens the susceptibility of the TRFIA to matrix interference [21,23].

This is the first report describing an Eu^{3+} fluorescent lanthanides-based TRFIA with high sensitivity and cost-efficiency for detection of tylosin and tilmicosin in edible tissues of food-producing animals. Comparisons are made between the TRFIA and conventional HPLC and ELISA methods. Finally, TRFIA was validated by analysis of swine muscle and liver samples.

1 Materials and methods

1.1 Materials and reagents

Tylosin, tilmicosin, desmycosin (DES), *O*-carboxymethoxylamine hemihydrochloride (CMO), spiramycin, erythromycin, kitasamycin, azithromycin, josamycin, oleandomycin, roxithromycin, avermectin, ivermectin, ovalbumin (OVA), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). *N*′-[*p*-Isothiocyanatobenzyl]-diethylene-triamineN^1,N^2,N^3,N^4-tetraacetate-Eu^{3+}(DTTA-Eu^{3+}) was from the Tianjin Radio-Medical Institute (Tianjin, China). Anti-tylosin monoclonal antibody (anti-tylosin MAb), DES-CMO-BSA and DES-CMO-OVA were obtained from Huazhong Agricultural University (Wuhan, China). A mouse monoclonal antibody isotyping kit was purchased from Southern Biotech Co. (Beijing, China). Sephadex 6B and G-50 were obtained from Pharmacia (Uppsala, Sweden). Buffers were prepared in our laboratory. All chemicals and reagents were of analytical grade or better, and the percentage concentrations are reported by weight unless otherwise specified.

1.2 Labeling of MAb with europium chelate

Europium-labeled MAbs (tracers) were prepared as described in the literature [24]. Briefly, 1 mg anti-tylosin MAb and 1 mg DTTA-Eu^{3+} were mixed in 50 mmol L^{-1} sodium carbonate buffer (pH 9.6) in an amber bottle and then kept at 4°C with stirring for over 24 h. The mixture was purified by a Sephadex 6B/G-50 column and 0.1% (w/v) BSA was added to the collected MAb-DTTA-Eu^{3+}. This mixture of MAb-DTTA-Eu^{3+} and BSA was ready for use and can be preserved for one year when stored at −20°C.

1.3 Pre-treatment of samples

Samples, such as porcine muscles, porcine liver, chicken muscle, chicken liver and fish, obtained at a local supermarket, were analyzed by HPLC for the tylosin and tilmicosin contents. The negative control was samples without tylosin and tilmicosin contamination, as identified by an HPLC test. The sample (2 ± 0.005) g was homogenized, mixed with 4 mL ethyl acetate, followed by 2 min vortexing and then 10 min centrifugation at 2000 r min^{-1}. The supernatant was gathered and 4 mL ethyl acetate was added to the pellet and the above treatment was repeated. The supernatant was evaporated in a water bath under a gentle nitrogen flow at 60°C. The extractions were resuspended in 1 mL of 10 mmol L^{-1} PBS (pH 7.4) and mixed thoroughly before TRFIA and ELISA analysis. The samples were pretreated for HPLC analysis, as described previously [25].

1.4 The competitive TRFIA

The 96-well Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with conjugates (DES-CMO-BSA, 1 mg L^{-1}) diluted in carbonate buffer (50 mmol L^{-1}, pH 9.6), followed by blocking for 2 h at 37°C with a blocking buffer (50 mmol/L Tris-HCl, pH 8.0, containing 0.9% NaCl, 0.5% BSA, 0.4% stabilizing reagent, and 0.04% NaN_3). The plate was then washed three times with a washing buffer (10 mmol L^{-1} Tris-HCl, pH 8.0, containing 0.9% NaCl and 0.1% Tween 20). Subsequently, 50 μL standard solutions or samples and 50 μL europium-labeled MAbs diluted with assay buffer were added to the plates. After incubation at 37°C for 1 h, the plates were washed six times, and an enhancement solution (0.1 mol L^{-1} potassium biphthalate-acetic acid buffer containing 15 μmol L^{-1} β-naphthoyltrifluoroacetone and 0.1% (w/v) Triton X-100) was added to each well. The plates were then shaken for 5 min for reaction and then rested for 10 min. The fluorescence was measured using a VICTOR^2 multilabel counter (PerkinElmer Wallac, Turku, Finland) at excitation wavelength 340 nm and emission wavelength 615 nm, with a delay time of 400 μs. The tylosin and tilmicosin contents in the samples were calculated from standard curves. The half-maximal inhibition concentration (IC_{50}) was determined as a measure of the sensitivity of the TRFIA.

1.5 Comparison of the TRFIA with ELISA and HPLC

To further demonstrate the capability and accuracy of the TRFIA, a comparison between TRFIA, ELISA and HPLC

was conducted using samples (liver and muscle) from an animal-feeding experiment. Duroc castrated pigs with weights of 13–15 kg were kept separately in an animal facility for one week. Eight pigs were randomly divided into control and test groups. The control group (n = 1) was under mock intramuscular treatment with another drug. The test group (n = 3) was treated by tylosin intramuscularly at a dose of bodyweight (BW) 10 mg kg^{-1} daily for 5 continuous days. The animals were euthanized at days 0, 1, and 3 of withdrawal after tylosin treatments. The muscle and liver samples were collected separately and stored at −20°C until analysis by TRFIA, ELISA and HPLC.

2 Results and discussion

2.1 Preparation of optimum working solutions for antibodies

Tylosin or tilmicosin in a series of dilutions were added to the coated plate. As shown in Figure 1, the anti-tylosin MAb solutions diluted at 1∶4000–1∶10000 produced inhibitory rates of 50%–30%. The working concentration for the anti-tylosin MAb was 1∶5000.

2.2 Cross-reactivities of MAb

The cross-reactivities between tilmicosin, tylosin, and their structural analogues (desmycosin, 5-*O*-mycaminosyltylonolide, spiramycin, erythromycin, kitasamycin, azithromycin, josamycin, oleandomycin, roxithromycin, avermectin, ivermectin) were tested to assess specificity. As shown in Table 1, the MAb showed cross-reactivity towards tylosin (100%), tilmicosin (51%), desmycosin (176%), and 5-*O*-mycaminosyltylonolide (4.5%), but did not generate significant cross-reactivity (<0.1) with the other antibiotics listed above. Thus, the TRFIA could be employed as a screening method for multiresidue detection.

2.3 The standard curve for the TRFIA

The optimum conditions for TRFIA were a coating antigen (DES-CMO-OVA) concentration of 1 mg L^{-1} and a MAb dilution ratio of 1∶8000, which generated an optimal linear portion of the response curve. These conditions were adopted for subsequent experiments. The standard curves plotted by TRFIA analysis showed that the working concentration ranged from 0.01 to 100 ng mL^{-1} (Figure 2). Good linearity was obtained in the range of 0.08 to 25 ng mL^{-1}, with acceptable correlation coefficients (R^2=96.83%–99.19%). With regard to assay sensitivity, IC_{50} values were calculated to be 0.89 and 1.73 ng mL^{-1} for tylosin and tilmicosin, respectively. The limit of detection (LOD) was expressed as mean + 3 × standard deviations [22]. Blank samples were determined by repeated analysis (n = 20) of tylosin/tilmicosin-free samples. The LODs of the TRFIA for tylosin and tilmicosin were 0.03 and 0.05 ng mL^{-1}, respectively. The definition for limit of quantification (LOQ) was the tylosin/tilmicosin concentration of samples with a B/B_0 ratio of approximately 0.8 and coefficient of variation (CV) less than 20% [22]. The LOQ for tylosin was 0.07 ng mL^{-1}, with CVs of 4.7, while the LOQ for tilmicosin was 0.11 ng mL^{-1}, with CVs of 5.3.

Table 1 Cross-reactivity of macrolide antibiotics by the TRFIA based on MAb

Competitor	IC_{50} (ng mL^{-1})	Cross-reactivity (%)
Tylosin	0.98	100
Tilmicosin	1.92	51
Desmycosin	0.56	176
5-*O*-mycaminosyltylonolide	21.78	4.5
Spiramycin	>1000	<0.1
Erythromycin	>1000	<0.1
Kitasamycin	>1000	<0.1
Azithromycin	>1000	<0.1
Josamycin	>1000	<0.1
Oleandomycin	>1000	<0.1
Roxithromycin	>1000	<0.1
Avermectin	>1000	<0.1
Ivermectin	>1000	<0.1

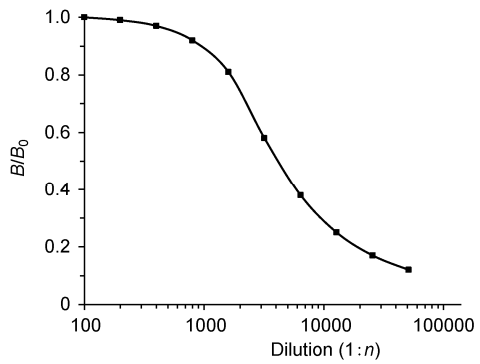

Figure 1 Dilution curve of MAb by TRFIA.

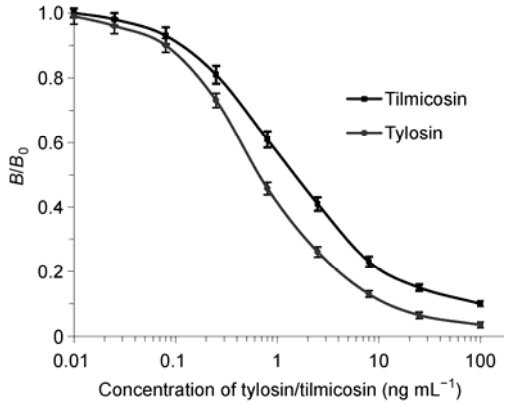

Figure 2 Calibration curve of tylosin/tilmicosin TRFIA.

2.4 Recovery and reliability of the TRFIA assay

The MRL for tylosin or tilmicosin is 100 ng g^{-1} in muscle and liver and 50 ng g^{-1} in egg [1]. The recoveries and repeatability of various biological matrices spiked with tylosin or tilmicosin at levels of 0.5 × MRL, 1 × MRL, and 2 × MRL are listed in Table 2. Close correlations were obtained between the measured values and the fortified concentrations at the three spiked levels. The recovery for tylosin ranged between 73.6% and 108.5% and that for tilmicosin ranged between 75.1% and 120.5%. The repeatability of the assay was validated by calculating CVs, which were less than 15.6%.

2.5 Comparison between TRFIA, ELISA and HPLC

Table 3 displays a summary of the results from TRFIA, HPLC and ELISA analyses, which reveals that 1–3 withdrawal days were needed for the attenuation of drug concentration to the MRLs levels (100 ng g^{-1}) in both swine muscle and liver. For quantitative analyses, TRFIA, ELISA and HPLC methods showed good correlation coeficients (R^2>0.99) in a side-by-side comparison. However, the TRFIA results are closer to HPLC results than to ELISA results for positive tylosin samples collected intraday and at the first day of withdrawal.

3 Conclusions

In this study, a simple and efficient TRFIA method has been established, which enables the analysis of tylosin and tilmicosin in edible animal tissues. The TRFIA method with europium tracer has a significantly increased sensitivity compared with the conventional ELISA. To validate the new method, comparisons were made between TRFIA, HPLC and ELISA in animal experiments, by which good correlations (R^2 > 0.99) were obtained and the new method was validated. The detection limits, CVs, and recoveries satisfy the standards of the USA, EU, Canada, and China. Therefore, the new TRFIA described here is a rapid, accurate, and economical method for detecting tylosin, tilmicosin and their metabolites, simultaneously, in animal tissues.

Table 2 Recoveries of tylosin and tilmicosin spiked in tissues by the TRFIA (n = 15) [a)]

Sample	Spiked level (ng g^{-1})	Tylosin		Tilmicosin	
		Mean recovery (ng g^{-1})	CV (%)	Mean recovery (ng g^{-1})	CV (%)
Porcine muscle	50	94.6	9.6	88.7	12.5
	100	73.6	5.3	108.7	15.6
	200	95.3	4.8	112.5	7.9
Porcine liver	50	103.7	12.4	95.4	11.3
	100	79.4	10.3	106.8	10.8
	200	86.5	8.4	112.1	15.3
Chicken	50	87.4	7.5	85.3	9.7
	100	97.6	4.7	99.8	11.6
	200	78.2	13.2	76.2	10.7
Chicken liver	50	102.6	10.6	93.2	14.8
	100	84.2	6.4	75.1	8.6
	200	108.5	5.9	84.9	5.6
Fish	25	96.3	9.7	100.8	12.8
	50	79.2	11.6	120.5	7.3
	100	88.3	10.5	96.3	13.5

a) The test was repeated three times with five replicates per concentration.

Table 3 Result analyses of HPLC, ELISA and TRFIA, for the detection of tylosin in swine muscle and liver samples from animal experiments (mean ± SD, n = 5)[a)]

Withdrawals (d)	Samples	HPLC (ng g^{-1})	ELISA (ng g^{-1})	TRFIA (ng g^{-1})
0	Muscle	186.4 ± 13.8	172.9 ± 23.5	185.2 ± 9.3
	Liver	487.5 ± 48.6	448.3 ± 50.4	485.6 ± 27.6
1	Muscle	78.3 ± 13.8	67.5 ± 4.2	77.9 ± 10.5
	Liver	136.2 ± 15.2	119.7 ± 16.2	132.2 ± 10.6
3	Muscle	<50	<20	18.7 ± 4.3
	Liver	61.4 ± 9.7	53.2 ± 12.8	61.8 ± 6.5

a) SD means of standard deviation. LOD of tylosin by HPLC method was 50 ng g^{-1}.

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